On the Synthesis of a Hemopeptide

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Cytochrome c is an enzyme which contains one mole of heme as a prosthetic group. The protein part is joined to the heme by two thioether linkages,[1] involving the sulfur of two cysteine residues, as is shown in Fig. 1. The imidazolyl group of the histidine residue is also linked to the iron of heme to form a hemochromogen.[2-4] The heme-containing fragment (hemopeptide) of horse-heart cytochrome c was first isolated by Tsou,[5,6] while the structure shown in Fig. 1 was determined by Tuppy et al.[7-9] After the complete hydrolysis of cytochrome c, there remains a kind of porphyrin which is the so-called "porphyrin c" and which has been considered to be an addition compound of protoporphyrin with two cysteines, as in the case of cytochrome c. More recently, the amino acid sequences of many different hemopeptides which have been isolated from the cytochrome c's of members of various species have been elucidated (Table I). Although the amino acid sequences of those hemopeptides are usually different from one another, they are represented by the general structure -CyS-X-Y-CyS-His-, where X and Y represent two other amino acid residues. It is very interesting that the hemopeptide obtained from beef-heart cytohrome c possesses a much stronger peroxidase-like activity than that of the parent enzyme.[10]

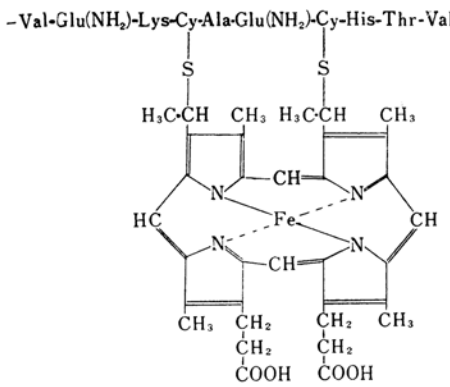

Fig. 1. Hemopeptide obtained from bovine heart cytochrome c by peptic digestion.

The present work, which adopts a synthetic approach, is on the relationship between the structure and the function of the hemopeptide. Two simplified peptides, cysteinyl-glycyl-glycyl-cysteine and cysteinyl-glycyl-glycyl-cysteinyl-histidine, were selected as the peptide moieties for the synthesis of model hemopeptide.

1) H. Theorell, *Biochem. Z.*, **298**, 242 (1928).
2) H. Theorell and A. Akesson, *J. Am. Chem. Soc.*, **63**, 1804 (1941).
3) K. G. Paul, *Acta Chem. Scand.*, **5**, 389 (1951).
4) A. Ehrenberg and H. Theorell, ibid., **9**, 1193 (1955).
5) C. L. Tsou, *Biochem. J.*, **49**, 362 (1951).
6) C. L. Tsou, ibid., **49**, 367 (1951).
7) H. Tuppy and G. Bode, *Monatsh.*, **85**, 1024 (1954).
8) H. Tuppy and G. Bode, ibid., 85, 1182 (1954).
9) H. Tuppy and S. Paléus, *Acta Chem. Scand.*, **9**, 353 (1955).
10) S. Paléus, A. Ehrenberg and H. Tuppy, ibid., **9**, 365 (1955).

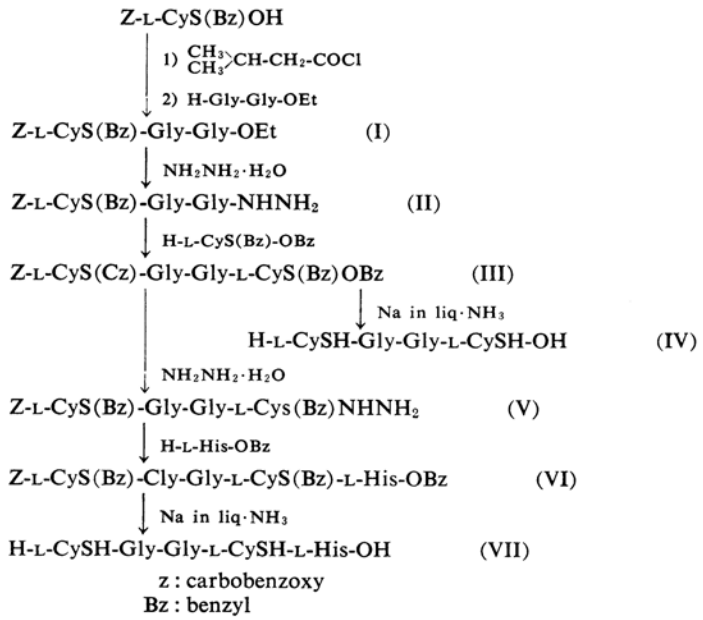

Fig. 2. Syntheses of H-L-CySH-Gly-Gly-L-CySH-OH and H-L-CySH-Gly-Gly-L-CySH-L-His-OH.

TABLE I. AMINO ACID SEQUENCES OF HEMOPEPTIDES OBTAINED FROM CYTOCHROME C

Species	Amino acid sequences
Horse[a]	-Lys-CyS-Ala-Glu(NH_2)-CyS-His-Thr-Val-Glu-
Pig[a]	
Bovine[b]	
Salmon[b]	
Human[c]	-Lys-CyS-Ser-Glu(NH_2)-CyS-His-Thr-Val-Glu-
Chicken[b]	-Lys-CyS-Ser-Glu(NH_2)-CyS-His-Thr-Val-Glu-
Silk Worm[d]	-Arg-CyS-Ala-Glu(NH_2)-CyS-His-Thr-Val-Glu-
Yeast[e]	-Arg-CyS-Glu-Leu-CyS-His-Thr-Val-Glu-
Pseudomonas fluorescens[f]	-Gly-CyS-Val-Ala-CyS-His-Ala-Ileu-Asp-
Rhodospillirum rubrum[g]	-Lys-CyS-Leu-Ala-CyS-His-Thr-Phe-Asp-

a) H. Tuppy and G. Bodo, *Monatsh.*, **85**, 1024, 1182 (1954).
b) H. Tuppy and S. Paléus, *Acta Chem. Scand.*, **9**, 353 (1955).
c) H. Matsubara and E. L. Smith, *J. Biol. Chem.*, **237**, 3575 (1963).
d) H. Tuppy, *Z. Naturforsh.*, **12B**, 784 (1957).
e) H. Tuppy and K. Dus, *Monatsh.*, **89**, 407 (1958).
f) R. P. Ambler, *Biochem. J.*, **82**, 309 (1962).
g) K. Levin, *Acta Chem. Scand.*, **15**, 1739 (1961).

The Synthesis of Peptide.—Carbobenzoxy-*S*-benzyl-L-cysteinyl-glycyl-glycyl-*S*-benzyl-L-cysteinyl-L-histidine benzylester (V) was synthesized as shown in Fig. 2. First, carbobenzoxy-*S*-benzyl-L-cysteinyl-glycyl-glycine ethylester was prepared according to the procedure of Hooper et al.[11] Although, in their paper, ethyl chloroformate was used for the preparation as a mixed anhydride-reagent, it has been found that the use of isovaleroyl chloride, instead of ethyl chloroformate, afforded a product with a higher melting point and a larger optical rotation than that which they reported. The improved procedure favored the succeeding reaction steps, and it was confirmed that the final melting point and the optical rotation of product III were much higher than those previously reported. The carbobenzoxy-tetrapeptide was successfully coupled with histidine benzyl ester by the azide procedure. Then, the carbobenzoxy-tetra- and penta-peptide benzylesters (III and VI respectively) were converted to free peptides (IV and VII) by treatment with sodium in liquid ammonia; they were then uesd in the preparation of the two hemopeptides.

The Preparation of Hemopeptide. — Theorell first demonstrated[1] that when protoporphyrin IX and cysteine were heated in mineral acid, a porphyrinc like compound was formed. Later, Zeile and Meyer[12] developed a practical way of preparing the same compound using hydrobrominated protoporphyrin as an intermediate. This was fused with excess cysteine hydrochloride without any solvent. Recently, Neilands and Tuppy[13] have published an improved method for the purification

11) K. C. Hooper, H. N. Rydon, J. A. Schofield and G. S. Heaton, *J. Chem. Soc.*, **1956**, 3148.
12) K. Zeile and H. Meyer, *Z. Physiol. Chem.*, **262** 178 (1939/40).
13) J. B. Neilands and H. Tuppy, *Biochim. Biophys. Acta*, **38**, 351 (1960).

of the product, producing a crystallizable porphyrin c.

No good procedure has as yet been published for the condensation of porphyrin with peptide, which contains two cysteine residues in the molecule. For the present work, the procedures reported for porphyrin c formation could not be used, because they require excess peptide at a high temperature, and this condition may cause a partial decomposition of the peptides. For the addition reaction of free cysteinyl peptide with hydrobrominated protoporphyrin, various alkaline conditions were tested at room temperature. Aqueous solutions of sodium hydroxide, carbonate or bicarbonate caused a partial decomposistion of sulfhydryl groups before the addition reaction was complete. Liquid ammonia, in which the free peptides were generated by sodium hydrogenolysis, was also not suitable for the reaction, because aminolysis by the bromide was faster than the formation of thioether linkages. Finally, the direct addition of protoheme to free cysteinyl peptide in liquid ammonia was tested, whereupon some kind of addition product was obtained. Although the mode of the formation of the complex between peptide and heme is still unknown, only the histidine containing pentapeptide afforded a reasonable amount of product.*

The crude product synthesized from peptide VII was dialyzed against deionized water to remove the unreacted peptide, and then it was subjected to counter-current distribution in a mixture of chloroform, methanol and 0.1 N hydrochloric acid (2 : 2 : 1). This procedure gave two brown fractions, as is shown in Fig. 3. The distribution coefficient of band I, shown in Fig. 3, agreed with that of hemin, and no amino acid was detected by the paper chromatography of a hydrolyzate of this fraction. On the other hand, glycine, histidine and a small amount of cystine were detected in a hydrolyzate of band II.

When the apparent molecular weight of the synthetic hemopeptide was calculated separately from the iron and glycine contents, values of 1030 and 1120 were obtained respectively. Theses values are in good agreement with the theoretical value of 1127.

No sulfhydryl group was detected by the Folin method[14] in the purified hemopeptide. Disulfide bonds were estimated by polarography, using the diffusion current at the half-wave potential in a range proportional to the concentration of cystine (Fig. 4), and it was concluded that the hemopeptide still contained three per cent of the total cysteine residues as disulfide bonds.

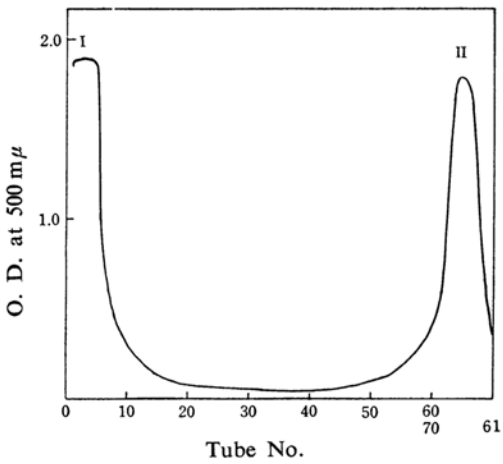

Fig. 3. Counter current distribution pattern of the crude product.
I; Hemin. II; Hemopeptide.
System; methanol : chloroform : 0.1N hydrochloric acid (2 : 2 : 1).

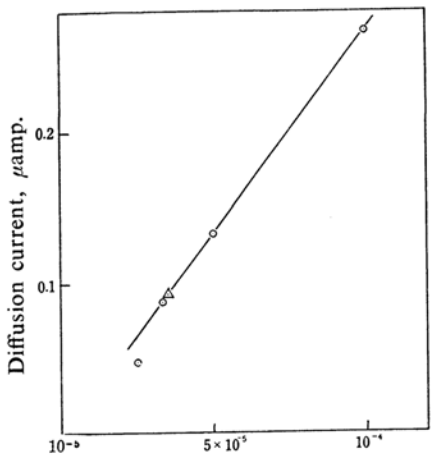

Fig. 4. Rotation between intensity of diffusion current at half-wave potential and concentration of cystine in 0.1 M ammonia buffer, pH 9.
⊙ Standard curve of mixture of cystine and hemin (1 : 1).
△ Intensity of diffusion current at half-wave potential of 9.2×10^{-4} M synthetic hemopeptide.

The Properties of Hemopeptide.—The synthetic hemopeptide was soluble in a water-methanol or water-acetone mixture (1 : 2 v/v), and in water at alkaline pH values. It was insoluble in chloroform, ether and water at neutrality. Although the infrared spectrum

* The authors wish to thank to Dr. Junzo Noguchi of Hokkaido University for his helpful discussion and advice concerning the coupling method.

14) B. Kassell and E. Brand, *J. Biol. Chem.*, **125**, 115 (1938).

resembled that of natural hemopeptide from beef-heart cytochrome c by peptic degradation[15] (abbreviated in this paper as hemopeptide N), the visible absorption spectrum of the synthetic compound was rather more similar to that of hemin than to that of hemopeptide N, as Figs. 5 and 6 show.

The peroxidative activities of hemopeptide N, synthetic hemopeptide, hemin and ferric chloride were compared by Shibata's method, using leuco malachite green as a hydrogen doner. As is shown in Fig. 7, the color of malachite green appeared after the induction period. In the case of ferric ion, no development of color was observed, even after 15 hr. This indicates that the catalytic activity of the synthetic hemopeptide was about 100 times as strong as that of hemin, but it was still inferior to that of natural hemopeptide N (Table II). This could be attributed to differences in the amino acid sequences and/or in the modes of the combination of the peptide and hemin.

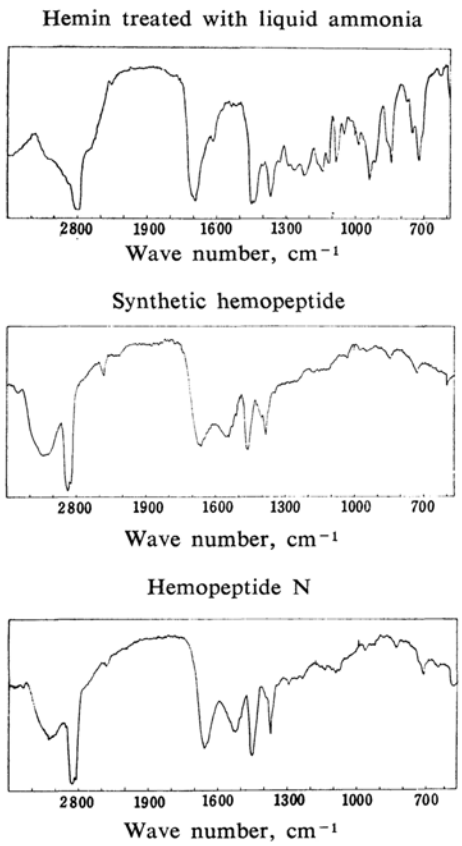

Fig. 5. Infrared absorption spectra recorded on a Shimadzu IR spectrophotometer in Nujol suspension.

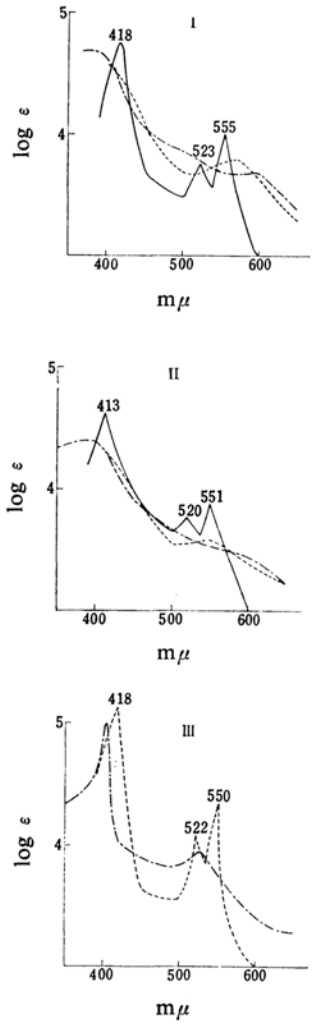

Fig. 6. Visible absorption spectra recorded on Cary model 14 recording spectrophotometer. I; Hemin II; Synthetic hemopeptide III; Hemopeptide N in 1/15 M phosphate buffer, pH 8.
— — — No addition
- - - - Reduced by sodium hydrosulfite
——— Pyridine hemochlomogen

After most of this work had been done, Sano[16,17] reported the interesting fact that freshly-prepared protoporphyrinogen combines with various sulfhydryl compounds more readily under mild conditions, and it was demonstrated that a porphyrin c-like compound is readily prepared in this manner by combination with cysteine. This procedure should be quite superior to any previous methods so far published for hemopeptide formation. Studies along these lines are in progress.

15) E. Margoliash. N, Frahwirt and E. Wiener, *Biochem. J.*, **71**, 559 (1958).

16) S. Sano, *Acta Haem. Japan*, **24**, 621 (1961).
17) S. Sano and S. Granick, *J. Biol. Chem.*, **236**, 1173 (1961).

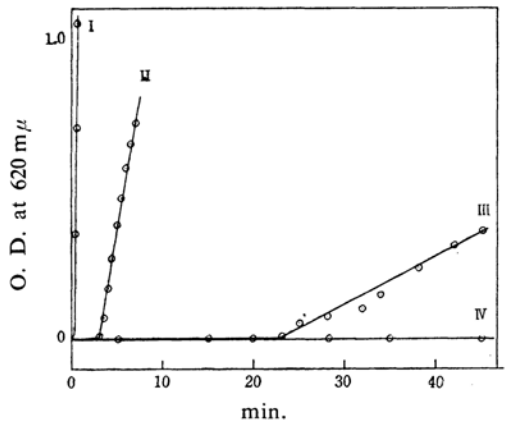

Fig. 7. Peroxidative activity by Shibata's method.
I; Hemopeptide N II; Synthetic hemopeptide III; Hemin IV; Ferric chloride

TABLE II. REACTION VELOCITIES AND INDUCTION PERIODS OF HEMIN, FERRIC ION AND HEMOPEPTIDES

Material	K*	Induction period, min.
Hemopeptide N	23.9	0.25
Synthetic hemopeptide	1.1	3
Hemin	0.009	23
Ferric ion	—	—

* Moles of malachite grean per mole of sample per min. These values were calculated after the respective induction periods.

Experimental*

Carbobenzoxy-*S*-benzyl-L-cysteinyl-glycyl-glycine Ethyl Ester (I).—Isovaleroyl chloride (6 g.; 0.05 mol.) was slowly stirred into a solution of *S*-benzyl-*N*-carbobenzoxy-L-cysteine (17.3 g.; 0.05 mol.) and triethylamine (6.9 ml.) in chloroform (175 ml.) at −5°C. After 1.5 hr., a mixture of glycyl-glycine ethyl ester hydrochloride (11.8 g.; 0.06 mol) and triethylamine (8.2 ml.) in chloroform (125 ml.) was stirred into the reaction mixture at −5°, and then the mixture was stored overnight in a refrigerator. The solution was washed successively with dilute hydrochloric acid, water, a sodium bicarbonate solution, and water, and then dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure, and the residual oil was redissolved in ethyl acetate (90 ml.). The slow addition of ethyl ether (150 ml.) to this solution resulted in the precipitation of a crystalline product (21 g.). The recrystallization of this material from ethyl acetate gave long needles; m. p. 118°C, yield 74% (18 g.). Hooper et al.[11] reported a m. p. of 114~115°C.

Carbobenzoxy-*S*-benzyl-L-cysteinyl-glycyl-glycine Hydrazide (II).—Hydrazine hydrate (7 ml.; 0.11 mol.) was added to a solution of I (17.5 g.; 0.036 mol.) in ethanol (250 ml.), and the solution was kept overnight at room temperature. The precipitate which formed was collected by filtration. The product was recrystallized from ethanol (200 ml.) and water (50 ml.) as needles; m. p. 173°C, yield 92% (15.7 g.). Hooper et al. reported a m. p. of 164°C.

Carbobenzoxy-*S*-benzyl-L-cysteinyl-glycyl-glycyl-*S*-benzyl-L-cysteine Benzyl Ester (III).—Triethylamine (7 ml.) was added to a suspension of *S*-benzyl-L-cysteine benzyl ester *p*-toluenesulfonate (23.7 g:, 0.05 mol.) in a mixture of ethyl ether (250 ml.) and water (40 ml.). The free benzyl ester which formed was extracted thoroughly with ethyl ether, and the ether extract was dried over anhydrous sodium sulfate. A solution of II (23.7 g.; 0.05 mol.) in a mixture of acetic acid (220 ml.), 2N hydrochloric acid (17 ml.) and water (220 ml.) was treated with sodium nitrite (3.5 g.; 0.05 mol.) at −5°C. The azide formed was extracted three times with cold ethyl ether (total: 600 ml.). The combined ethyl ether layer was washed with cold water 100 ml. and dried over anhydrous sodium sulfate at −5°C. The dry ether solution was added to the solution of *S*-benzyl-cysteine benzyl ester prepared as described above, and the mixture was stored overnight in a refrigerator (0~5°C). The precipitate which formed was collected by filtration and recrystallized from ethanol (500 ml.) as needles; yield 83% (3.17 g.); m. p. 178~179°C; $[\alpha]_D^{15}$ −40.7° (*c* 2.73 in pyridine). Hooper et al.[11] reported a m. p. of 175°C and $[\alpha]_D^{20}$ −29.6° in pyridine.

Found: C, 62.81; H, 5.74; N, 7.57; S, 8.40. Calcd. for $C_{39}H_{42}O_7N_4S_2$ C, 63.05; H, 5.70; N, 7.54; S, 8.63%.

Carbobenzoxy-*S*-benzyl-L-cysteinyl-glycyl-glycyl-*S*-benzyl-L-cysteine Hydrazide (V).—A solution of III (10 g.; 13.5 mol.) in a mixture of dioxan (150 ml.) and ethanol (50 ml.) was treated with hydrazine hydrate (30 ml.; 0.5 mol.). After 24hr. at room temperature, the solvent was removed at 30°C under reduced pressure, and the residue was crystallized by adding ethanol (160 ml.). The product was recrystallized from ethanol (160 ml.) and water (40 ml.) as needles; yield 77% (7.1 g.); m. p. 162°C.

Found: C, 57.28; H, 5.82; N, 12.21. Calcd. for $C_{32}H_{38}O_6N_6S_2$ C, 57.64; H, 5.74; N, 12.60%.

Carbobenzoxy-*S*-benzyl-L-cysteinyl-glycyl-glycyl-*S*-benzyl-L-cysteinyl-L-histidine Benzyl Ester (VI).—Substance V (5 g.; 7.5 mmol.) was dissolved in a mixture of acetic acid (40 ml.) and water (40 ml.), and then 2N hydrochloric acid (7.5 ml.) was added to the solution. The mixture was carefully treated with sodium nitrite (0.52 g.; 7.5 mmol.) at −5°C, and the azide which formed was extracted with cold chloroform total: 300 ml.). The azide solution was washed with ice water, and then dried over sodium sulfate at −5°C. The dry chloroform solution was added to a cold solution of L-histidine benzyl ester di-*p*-toluenesulfonate (6.7 g.; 8.8 mmol.) and triethylamine (3.1 ml.) in chloroform (50 ml.), and the reaction mixture was allowed to stand overnight in a refrigarator. It was then washed with water and a sodium bicarbonate solution. During this procedure, some products appeared as

* The melting points given here are uncorrected.

crystals. Petroleum ether (b. p. 60~80°C) was added to the mixture to complete the precipiation, and the preduct was collected by filtration and recryllized from ethanol (24 ml.) and water (6 ml.); yield 60% (4 g.); m. p. 178°C; $[\alpha]_D^{16}$ −54.3° (*c* 0.98 in dioxan).

Found: C, 61.25; H, 5.59; N, 10.96; S, 7.65. Calcd. for $C_{45}H_{49}O_8N_7S_2$ C, 61.41; H, 5.61; N, 11.14; S, 7.29%.

Hemopeptide.—A solution of substance VI (880 mg.; 1 mmol.) in liquid ammonia (500 ml.) was treated wtih approximately 200 mg. of sodium, in portions, until a permanent blue color appeared in the solution. Then 70 mg. more sodium and protohemin (652 mg.; 1 mmol.) were vigorously stirred into the reaction mixture. After the addition of a small amount of ammonium chloride to neutralize the excess sodium, the reaction mixture was stirred for 4hr. and then stored overnight in a dry ice bath. After the evaporation of the solvent, the product was dried under reduced pressure over sulfuric acid in a desiccaior. The residue was dissolved in water (50 ml.), and the aqueous solution was then dialyzed against running deionized water for 3 days. The lyophilization of the solution gave the sodium salt of the crude product as a brown-red prowder (yield, 930 mg.). The product (400 mg.). was subjected to counter-current distribution in a solvent system of chloroform, methanol and 0.1 N hydrochloric acid (2 : 2 : 1) (Fig. 3.). Band II (tubes 61~70) was concentrated to about 50 ml. at 40°C under reduced pressure. The residual solution was adjusted to pH 9 with a dilute aqueous solution of sodium hydroxide and then dialyzed through cellophane tubing against running deionized water. The final solution was lyophilized to yield the sodium salt of the hemopeptide; yield 163 mg.; m. p. above 260°C. For analysis, the hemopeptide (30 mg.) was dissolved in deionized water, and the solution was acidified with dilute hydrochloric acid to about pH 2. The solution was dialyzed against running deionized water, and the precipitates which formed were collected by centrifugation and dried in vacuo at 80°C.

Found: C, 49.91; H, 4.97; N, 13.66; S+Cl, 9.54; Fe, 5.42. Calcd. for $C_{50}H_{56}O_{10}N_{11}S_2Fe \cdot 2HCl$: C, 51.59; H, 5.02; N, 13.24; S+Cl, 11.60; Fe, 4.80%. Mol. wt. Found: 1030*; 1160**. Calcd.: 1164.

Peroxidative Activity.—The peroxidative activities of the synthetic hemopeptide and other substances were assayed, using leuco malachite green as a hydrogen donor, by the method of Shibata[19] as follows: The reaction system was composed of 2.0 ml. of a 0.2 M phosphate-hydrochloric acid buffer (pH 3.1), 8.82×10^{-2} mol. hydrogen peroxide, 6.77×10^{-3} mol. leuco malachite green and 1.485×10^{-5} mol. synthetic hemopeptide or other substances in a total volume of 3.5 ml. of water. The reaction was started by adding the hydrogenperoxide, and incubation was carried out at 15°C. The activity was estimated spectrophotometrically by measuring the increase in optical density at 620 mμ due to the oxidation of leuco malachite green to malachite green. The activity was expressed as the amount of malachite green formed per minute after the induction period.

Summary

Carbobenzoxy-*S*-benzyl-L-cysteinyl-glycyl-glycyl-*S*-benzyl-L-cysteinyl-L-histidine benzylester has been synthesized, and then it has been hydrogenated to the free peptide by treatment with sodium in liquid ammonia. The free peptide has been coupled with protohemin in the liquid ammonia to form a hemopeptide, which has then been purified by counter-current distribution. The visible and infrared absorption spectra of the product have been compared with those of natural hemopeptide, which was obtained from beef-heart cytochrome c by peptic degradation. The peroxidative activity of the synthetic hemopeptide is about 100 times as strong as that of hemin, but it is inferior to that of natural hemopeptide.

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* Calculated from the iron content.

** Calculated from the glycine content in a hydrolyzate by the improved method of Levy.[18]

18) A. L. Levy, *Nature*, **174**, 126 (1954).

19) Y. Nakamura, T. Samejima, K. Kurihara, M. Tohjo and K. Shibata, *J. Biochem.*, **48**, 862 (1960).